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In re Application of:

MINAMITAKE, et al.

Application Number: 10/500,561

I. A. Filing Date: May 21, 2003

For: MEDICAL COMPOSITIONS CONTAINING GHRELIN

Group Art Unit: To be Assigned

Examiner: To Be Assigned

SUBMISSION OF CERTIFIED COPY OF PRIORITY DOCUMENT

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Commissioner for Patents

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Alexandria, VA 22313-1450

Sir:

Applicants respectfully submit an English translation of a certified copy of Japanese Patent Application No. JP2002-146155, filed May 21, 2002, in connection with the above-identified patent application ("the Application"). Applicants claim foreign priority benefits under 35 U.S.C. § 365(b) from the Japanese Patent Application in the Application, which is a National Stage Application of International Patent Application No. PCT/JP03/06349, filed May 21, 2003. A declaration stating that the translation is a true translation of the Japanese Patent Application into the English language is submitted herewith.

It is believed that no fees are due in connection with the submission of these documents. However, if any fees are determined to be due, the Commissioner is hereby authorized to charge these fees to the undersigned's Deposit Account No. 50-0206.

Respectfully submitted,

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DECLARATION

In the matter of an Application for Letters Patent by
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I, Osamu KUSAMA, Patent Attorney, whose full post office
address is 7th Floor, Iwata Bldg., 5-12, Iidabashi 4-chome,
Chiyoda-ku, Tokyo 102-0072, Japan, do solemnly and sincerely
declare as follows:

1. I am well acquainted with Japanese and English language.
2. The following is the true translation into English language
of the PCT application No. JP2002-146155 by SUNTORY LIMITED with
the Receiving Office / The Japanese Patent Office on May 21,
2002 in respect of an Application for Letters Patent.

And I make this solemn declaration conscientiously
believing the same to be true.

Declared at Tokyo, Japan
This 29th day of July, 2004.


Osamu KUSAMA
KUSAMA PATENT OFFICE

JP2002-146155

(Translation)

JAPAN PATENT OFFICE

5 This is to certify that the annexed is a true copy of the following application
as filed with this office.

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10 Application Number: Patent Application No. 2002-146155
[ST.10/C]: [JP2002-146155]

Applicant(s): DAIICHI SUNTORY PHARMA CO., LTD.

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Shinichiro Ohta
Commissioner, Japan Patent Office

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[List of the Documents]

 [Item] Specification 1

 [Item] Figure 1

 [Item] Abstract 1

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【Name of the Document】 DESCRIPTION

【Name of the Invention】 PHARMACEUTICAL COMPOSITION CONTAINING GHRELIN

【Claims】

5 【Claim 1】 A pharmaceutical composition stably containing
ghrelin or its derivative, wherein pH of an aqueous solution dissolving
the ghrelins is from 2 to 7.

 【Claim 2】 A pharmaceutical composition according to claim 1,
wherein said aqueous solution having pH of from 2 to 7 is buffer.

10 【Claim 3】 A pharmaceutical composition according to claim 2,
wherein the buffer agent is one or more selected from the group consisting
of glycine, acetic acid, citric acid, boric acid, phthalic acid, phosphoric
acid, succinic acid, lactic acid, tartaric acid, carbonic acid,
hydrochloric acid, sodium hydroxide and the salt thereof.

15 【Claim 4】 A pharmaceutical composition according to claim 2,
wherein the buffer solution is glycine hydrochloride buffer, acetate buffer,
citrate buffer, lactate buffer, phosphate buffer, citric acid-phosphate
buffer, phosphate-acetate-borate buffer or phthalate buffer.

20 【Claim 5】 A pharmaceutical composition according to any one of
claims 2 to 4, wherein concentration of the buffer agent in the solution
is in the range of from 0.1mM to 1000mM.

 【Claim 6】 A pharmaceutical composition according to any one of
claim 1 to 5, wherein the concentration of the ghrelins or derivative
thereof in the solution is in the range of 0.03nmol/mL to 6μmol/mL.

25 【Claim 7】 A pharmaceutical composition according to any one of
claims 1 to 6, wherein an anti-adsorbent is further contained.

 【Claim 8】 A pharmaceutical composition according to claim 7,
wherein the concentration of the anti-adsorbent is in the range of from
0.001% to 5%.

30 【Claim 9】 A pharmaceutical composition according to claim 7,
wherein the anti-adsorbent is surfactant.

 【Claim 10】 A pharmaceutical composition according to any one of
claims 1 to 9, wherein the composition is dissolving preparation in situ.

【Claim 11】 A pharmaceutical composition according to claim 10, wherein the dissolving preparation in situ is lyophilized preparation.

【Claim 12】 A pharmaceutical composition according to any one of claims 1 to 11, wherein the composition is injectable solution.

5 【Disclosure of the invention】

【0001】

【Technical Field】

The present invention relates to a pharmaceutical composition containing ghrelin or derivative thereof, which is an endogenous growth hormone secretagogue (GHS) to a growth hormone secretagogue-receptor (GHS-R) in a stable state.

10 【0002】

【Background Art】

Ghrelin, an endogenous growth hormone secretagogue (GHS) to growth hormone secretagogue receptor (GHS-R) which is one of orphan receptors, is a physiologically active peptide first isolated and purified from rat in 1999 (Kojima, et al., *Nature*, 402: 656-660, 1999). Thereafter, some ghrelins having same chemical structure of rat ghrelin have been isolated from vertebrates other than rat, such as human, mouse, pig, chicken, eel, bovine, equine, ovine, frog, trout and canine. The chemical structures of these ghrelins are listed in the following Table 1.

15 【0003】

20 【Table 1】

Human	GSS(n-octanoyl)FLSPEHQRVQQRKESKKPPAKLQPR GSS(n-octanoyl)FLSPEHQRVQQRKESKKPPAKLQPR
Rat	GSS(n-octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR GSS(n-octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR
Mouse	GSS(n-octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR
Porcine	GSS(n-octanoyl)FLSPEHQKVQQRKESKKPAALKPR
Bovine	GSS(n-octanoyl)FLSPEHQKLQRKEAKKPSGRLKPR
Ovine	GSS(n-octanoyl)FLSPEHQKLQRKEPKKPSGRLKPR
Canine	GSS(n-octanoyl)FLSPEHQKLQQRKESKKPPAKLQPR
Eel	GSS(n-octanoyl)FLSPSQRPOGKDKKPPRV-NH ₂
Trout	GSS(n-octanoyl)FLSPSQKPQVRQGGKPPRV-NH ₂ GSS(n-octanoyl)FLSPSQKPQGGKPPRV-NH ₂
Chicken	GSS(n-octanoyl)FLSPTYKNIQQQKGTRKPTAR GSS(n-octanoyl)FLSPTYKNIQQQKDTRKPTAR GSS(n-octanoyl)FLSPTYKNIQQQKDTRKPTARLH
Bullfrog	GLT(n-octanoyl)FLSPADMQKIAERQSQNKLRHGNM GLT(n-decanoyl)FLSPADMQKIAERQSQNKLRHGNM GLT(n-octanoyl)FLSPADMQKIAERQSQNKLRHGNM
Tilapia	GSS(n-octanoyl)FLSPSQKPQNKVKSSRI-NH ₂
Catfish	GSS(n-octanoyl)FLSPTQKPQNRGDRKPPRV-NH ₂ GSS(n-octanoyl)FLSPTQKPQNRGDRKPPRVG
Equine	GSS(n-butanoyl)FLSPEHHKVQHRKESKKPPAKLKPR

【0004】

(wherein, an amino acid residue is written by the one letter notation defined by IUPAC and IUC)

【0005】

5 These peptides are characterized by a specific structure due to acylation of hydroxyl group at the side chain of serine group (S) or threonine group (T) by fatty acid such as octanoic acid or decanoic acid, and there has never been isolated the physiologically active peptides having modifying hydrophobic group such as ghrelin. These new peptides exhibit

10 potent promoting effect for secretion of growth hormone, and it has become clear that these peptides perform for adjusting the secretion of growth hormone. Therefore, many researchers have great interest in physiologically active roll of ghrelin and for development of these peptides

as medicines (e.g., World Patent Publication WO 01/07475).

【0006】

It is known that the modifying hydrophobic group in ghrelin molecule has to be necessary for exhibiting the physiological effects (Kojima, et al., *Nature*, 402: 656-660, 1999). However, due to the non-existence of peptides like ghrelin having the modifying hydrophobic group in molecule at the hydroxyl group of side chain of specific amino acid residue, the stability of these peptides for development as medicines have never been studied.

10 【0007】

Incidentally, the compound to be developed as medicines has the various kinds of chemical structures, and because of these chemical structures, the compounds may easily degrade in the formulation process or in the storage process thereafter. The degradation reactions are hydrolytic cleavage, dehydration, isomerization, elimination, oxidization, reduction or photodegradation of the compound, and further, the chemical reaction of the compound with additives to be formulated with the compound. Therefore, it is very important to study and understand the varieties of the degradation reaction and the degrees thereof from the chemical structure of the compounds, for development of the compound as medicines, and consequence quality control thereof.

20 【0008】

It is well known that the stability of medicines may be greatly controlled by the ambient environmental condition, such as pH level of the environment. The influence of pH of the solution for the degradation rate of medicines in aqueous state has been studied, and pH profile of degradation rate of many medicines has been reported (e.g., Sumie Yoshioka, "*Stability of Medicines*" by Nankohdo, 1995).

25 【0009】

30 The physiologically active peptides or physiologically active proteins are inactivated and degraded by protease existing in the digestive organ, and it is difficult to develop the oral administrable composition

containing these peptides or proteins. Therefore, these peptides or proteins are prepared as an injectable composition for the clinical administration, and for this purpose the stability of these substances in the aqueous solution is very important for preparation of the liquid pharmaceutical formulations regardless of the dosage form such as solution form or soluble solution form in site.

【0010】

At present, pharmaceutical compositions containing various kinds of peptide or protein such as insulin, growth hormone, calcitonin, atrial natriuretic peptide, LH-RH (luteinizing hormone-releasing hormone) derivatives or adrenocorticotrophic hormone derivatives are on sale as medicines, and it is reported that the chemical changes of these peptides or proteins are deamidation, iso-aspartic acid formation, hydrolytic cleavage such as fragmentation, racemization, formation of disulfide bond or exchange reaction, β -elimination or oxidative reaction.

【0011】

These chemical changes exert an influence on the stability of the composition containing peptides or proteins, and the degrees of the degradation reaction of peptides or proteins is dependent on a pH value of the solution. For example, it is reported that the chemical structure of degradation products and the produced amount of the degradation products varied according to the pH value of the solution containing these peptides or proteins, such as LH-RH derivatives (Strickley et al., *Pharm. Res.*, 7, 530-536, 1990), human parathyroid hormone (Nobuchi et al., *Pharm. Res.*, 14, 1685-1690, 1997), hirudin (antithrombin substance: Gietz et al., *Pharm. Res.*, 15, 1456-1462, 1998), and human amylin derivatives (Hekmann et al., *Pharm. Res.*, 15, 650-659 1998).

【0012】

Ghrelin or its derivative of the present invention is a physiologically active peptide, and it is common to prepare an aqueous solution containing ghrelin as pharmaceutical composition for medicine. Though the stability of ghrelin in the aqueous solution is very important

for preparation of the pharmaceutical composition, there has never been any study of the stability of ghrelin in the aqueous solution. Ghrelin or its derivative has the specific modifying hydrophobic group in its molecule, that is, the side chained hydroxyl group of certain amino acid residue of ghrelin or its derivative is acylated by fatty acid. There has never been discovered a peptide like ghrelin having the specific modifying hydrophobic group in molecule, therefore, the common knowledge about the stability of ghrelin has also never been reported.

【0013】

That is, it is unknown about the stability, the chemical structure of degradation product and the mechanism of production of the degradation product of ghrelin. Further, it is unknown about the mechanism of degradation of modifying hydrophobic group of ghrelin, as well as the secondary degradation from the degradation product of ghrelin.

【0014】

【The problem to be solved in the Invention】

Under these circumstances, the objective of the present invention is to provide a pharmaceutical composition stably containing ghrelin or its derivative based on the knowledge obtained by the investigation of the chemical stability of ghrelin or its derivative having specific modifying hydrophobic group in the molecule.

【0015】

Through extensive investigations of the influence of pH in an aqueous solution containing ghrelin and the chemical structure of the degradation product from ghrelin, the present inventors discovered that, in an aqueous solution, ghrelin degraded to produce desacyl compound by hydrolytic cleavage of the specific modifying hydrophobic group and in addition, degraded to produce dehydroalanine compound by β -elimination of modifying hydrophobic group, consequently to produce the secondary degradation product due to the volatility of dehydroalanine compound, and these degradation were affected by pH value of the aqueous solution.

【0016】

Based on the results of the mechanism of degradation of ghrelin mentioned above, the present inventors further discovered that the pharmaceutical composition stably containing ghrelin could be obtained by adjusting pH of the solution with pH adjuster or buffer agent, and this stabilization effect could be obtained by various sorts of the buffer agent independent of their concentration or ghrelin concentration, and thus completed the present invention.

【0017】

[Means to solve the problem]

Accordingly, as one aspect of the present invention, it is provided a pharmaceutical aqueous composition stably containing ghrelin or its derivative, wherein pH of an aqueous solution dissolving the ghrelins is from 2 to 7.

【0018】

More specifically, a said pharmaceutical composition is preferably buffer, wherein pH of said aqueous solution is adjusted by various buffer solution to the range from 2 to 7. Accordingly, a pharmaceutical composition according to claim 2 is, a pharmaceutical composition according to claim 1, wherein the aqueous solution having pH of 2 to 7 is buffer solution, and present invention according to claim 3 is a buffer agent containing one or more selected from the group consisting of glycine, acetic acid, citric acid, boric acid, phthalic acid, phosphoric acid, succinic acid, lactic acid, tartaric acid, carbonic acid, hydrochloric acid, sodium hydroxide and the salt thereof. Further, a pharmaceutical composition according to claim 4 is the buffer solution selected from glycine hydrochloride buffer, acetate buffer, citrate buffer, lactate buffer, phosphate buffer, citric acid-phosphate buffer, phosphate-acetate-borate buffer or phthalate buffer.

【0019】

The present invention provides another embodiment as a pharmaceutical composition according to any one of claims 2 to 4, wherein concentration of the buffer agent in the solution is in the range of from

0.1mM to 1000mM, and a pharmaceutical composition according to any one of claim 1 to 5, wherein the concentration of the ghrelins or derivative thereof in the solution is in the range of 0.03nmol/mL to 6µmol/mL.

【0020】

5 The present invention, is designed to inhibit absorption of the peptide of the solution containing ghrelins having fatty acid having an adamantane skeleton as modifying hydrophobic group and therefore, present invention provides a pharmaceutical composition according to any one of claims 1 to 6, wherein an anti-adsorbent is further contained.

10 【0021】

Additionally, the present invention provides a pharmaceutical composition according to claim 7, wherein the concentration of the anti-adsorbent is in the range of from 0.001% to 5%.

【0022】

15 Also, the present invention provides dissolving preparation in situ stably containing phrelins and derivatives thereof when dissolved in water, and therefore, the invention claimed as claim 10 is a pharmaceutical composition according to any one of claims 1 to 9, wherein the composition is dissolving preparation in situ. An invention claimed as claim 11 is
20 a pharmaceutical composition according to claim 10, wherein the dissolving preparation in situ is lyophilized preparation.

【0023】

【Best Mode for Carrying Out the Invention】

25 The pharmaceutical composition of the present invention will now be explained more specifically as following.

30 The ghrelins to be used in the present invention are endogenous growth hormone secretagogues (GHS), which are peptides having effect of increasing the intracellular calcium ion concentration and inducing the secretion of growth hormone. The suitable ghrelins are those obtained from human, rat, porcine, chicken, eel, bovine, equine, ovine, frog, trout or canine. The chemical structures of these ghrelins are listed in the following Table 1.

【0024】

Therefore, the ghrelin derivatives of the present invention include the peptides listed in the above-mentioned Table 1, in which the amino acid sequence is modified by the insertion, addition and deletion of one or more amino acid; and/or the substitution by other amino acid to said amino acid sequence, and is modified chemically if necessary.

【0025】

The modifying hydrophobic group, which is a characteristic of the ghrelins, is not limited to octanoyl (C₈) group, and is a residue of fatty acid having 2 to 20, preferably 4 to 12 carbon atoms, such as hexanoyl (C₆) group, decanoyl (C₁₀) group or dodecanoyl (C₁₂) group. Further, the hydrophobic group is a residue of branched, saturated or unsaturated fatty acid, a residue of fatty acid having an aromatic group such as phenylpropionyl group, and an adamantane skeleton.

【0026】

Further, the ghrelin derivatives of the present invention include the peptides in which modifying hydrophobic group is bonded to amino acid chain by ester bond and having same physiologically activity as the ghrelins.

【0027】

The ghrelins to be used in the pharmaceutical composition of the present invention include free form peptides and salts thereof. The free form peptide and salt thereof can be reciprocally converted. The free form peptide can be converted to a pharmaceutically acceptable salt by reacting with an inorganic or an organic acid. The examples of the salt include the salt with the inorganic acid, such as carbonate, bicarbonate, hydrochloride, sulfate, nitrate or borate; and the salt with the organic acid, such as succinate, acetate, propionate or trifluoroacetate.

【0028】

Further, the salt with alkali metal such as sodium salt or potassium salt; the salt with alkali earth metal such as calcium salt or magnesium salt; the salt with organic amine such as triethylamine salt; and the salt with basic amino acid such as alginic acid salt is included. The peptides

of the present invention can exist as metal complex such as copper complex or zinc complex.

【0029】

Herein after and in claims, ghrelins and the derivatives thereof are
5 referred to as "the ghrelins".

【0030】

The origins and the manufacturing methods of the ghrelins are not limited in the present invention. The ghrelins obtained by chemical process, semichemical process, genetical process or combination process thereof,
10 and extraction from living body can be used in the present invention.

【0031】

The ghrelins to be used as raw materials for medicines are commonly supplied as lyophilized powder after purified by reverse liquid chromatography and so on.

15 【0032】

The aqueous solution or solution of the present invention are the solution used water as the solvent; however, other solvent can be used within a pharmaceutically acceptable range.

【0033】

20 The concentration of the ghrelins in the pharmaceutical composition of the present invention is not limited, and is preferably within a pharmaceutically acceptable range. The lower limit of concentration is the concentration wherein the ghrelins exhibit the pharmacologically activities, and the upper limit of concentration is the concentration
25 wherein the ghrelins can be dissolve in the aqueous solutions. The concentration commonly used as medicines such as 0.03nmol/mL to 6 μ mol/mL is preferable, and more preferably, the concentration of 0.03nmol/mL to 3 μ mol/mL is used.

【0034】

30 In the physiological composition of the present invention stably containing the ghrelins, the pH value of the solution is in the range of 2 to 7, more preferably 3 to 6. It was found out that the stable pH

value of the solution containing the ghrelins is in the range of 2 to 7. The adjustment of pH of the solution containing the ghrelins is conducted with pH adjuster or buffer agent.

【0035】

5 Examples of pH adjuster include hydrochloric acid, gluconic acid, sulfuric acid, sodium carbonate, sodium hydrate, aqueous ammonia, citric acid, monoethanolamine, lactic acid, acetic acid, fumaric acid, maleic acid, phosphoric acid, methanesulfonic acid, malic acid, and salt thereof.

【0036】

10 Examples of buffer agent include glycine, acetic acid, citric acid, boric acid, phthalic acid, phosphoric acid, succinic acid, lactic acid, tartaric acid, carbonic acid, hydrochloric acid, sodium hydroxide, and the salt thereof.

【0037】

15 Considering the stability of the ghrelins in the aqueous solution, it is desired that the fluctuation of pH values of the solution have to be reduced. Therefore, the pharmaceutical composition of the present invention is the solution having buffer capacity, that is, the buffer solution.

20 【0038】

 The buffer solution having the pH range wherein the degradation of the ghrelins is inhibited is used, and the solution having the pH range of 2 to 7, more preferably 3 to 6 is used. The suitable buffer solution is glycine hydrochloride buffer, acetate buffer, citrate buffer, lactate
25 buffer, phosphate buffer, citric acid-phosphate buffer (including McIlvaine buffer), phosphate-acetate-borate buffer (including Britton-Robinson buffer), and phthalate buffer. The examples of the components of each buffers include the buffer agents mentioned above.

【0039】

30 The concentration of pH adjuster is not limited and can be the concentration commonly used to adjust the solution with the desired pH range, and in general, the concentration of 0.01 to 1000mM is used.

【0040】

Further, the concentration of buffer agent is also not limited and can be the concentration maintaining the buffer capacity. Generally, the concentration of 0.01 to 1000mM, more preferably 1 to 100mM is commonly used.

【0041】

According to the present invention, the pharmaceutical composition stably containing the ghrelins in the aqueous solution is provided. The composition contains other additives in consideration of osmolality, solubility, low irritation of the solution, as well as antiseptis effect and prevention of absorption of the ingredient in the solution.

【0042】

In general, there is fear that in the pharmaceutical aqueous solution containing peptides or proteins, the peptides or proteins adsorb to vessels used in the process for producing the solution or during the administering of the solution, and therefore, the concentration of peptides or proteins decrease. In the case of the pharmaceutical composition of the present invention, it was confirmed that the ghrelins adsorb to glass vessels or polypropylene vessels in the range of the concentration of the ghrelins for medical use. Therefore, it is preferable to contain the anti-adsorbent to prevent the adsorption of the ghrelins to vessels.

【0043】

Examples of anti-adsorbent include surfactants, saccharides, amino acids and proteins.

The surfactant of the present invention include the surfactants listed in the "Handbook of PHARMACEUTICAL EXCIPIENTS" as well as the compounds having surface-active effects, and the suitable surfactant is select from these surfactants. Examples include quaternary ammonium salts, polyoxyethylene sorbitan fatty acid esters, sorbitan fatty acid esters, parabens, polyethylene glycols, phospholipids, bile acids, polyoxyethylene castor oils, polyoxyethylenes, polyoxyethylene polyoxypropylenes, polyalcohols, anionic surfactant, synthetic or

semi-synthetic polymers.

【0044】

The suitable quaternary ammonium salts include benzalkonium chloride, benzethonium chloride and cetylpyridinium chloride. The
5 suitable polyoxyethylene sorbitan fatty acid esters include polyoxyethylene sorbitan monolaurate (Polysorbate® 20 or Tween® 20), polyoxyethylene sorbitan monopalmitate (Polysorbate® 40 or Tween® 40), polyoxyethylene sorbitan monostearate (Polysorbate® 60 or Tween® 60), polyoxyethylene sorbitan tristearate (Polysorbate® 65 or Tween® 65),
10 polyoxyethylene sorbitan monooleate (Polysorbate® 80 or Tween® 80), and polyoxyethylene sorbitan trioleate (Polysorbate® 85 or Tween® 85).

The suitable sorbitan fatty acid esters include sorbitan monolaurate (Span® 20), sorbitan monopalmitate (Span® 40), sorbitan monostearate (Span® 60), sorbitan monooleate (Span® 80), sorbitan trioleate (Span® 85), and
15 sorbitan sesquioleate.

【0045】

The suitable parabens include methyl paraoxybenzoate, ethyl paraoxybenzoate, propyl paraoxybenzoate, butyl paraoxybenzoate, and isobutyl paraoxybenzoate. The suitable polyethylene glycols include
20 glycolfurol (glycolfurol 75), Mcrogol® 400 (polyethylene glycol 400), Mcrogol® 600 (polyethylene glycol 600), and Mcrogol® 4000 (polyethylene glycol 4000); the suitable phospholipids include refined soybean lecithin and refined yolk lecithin; and suitable bile acids include sodium desoxycholic acid.

25 【0046】

The suitable polyoxyethylene castor oils include polyoxyethylene castor oil, polyoxyethylene hydrogenated castor oil, polyoxyethylene hydrogenated castor oil 50, and polyoxyethylene hydrogenated castor oil 60. Examples of other polyoxyethylenes include polyoxyethylene oleyl
30 ether, polyoxyethylene stearyl ether, polyoxyethylene cetyl ether, and polyoxyethylene lauryl sulfate salt. The suitable polyoxyethylene polyoxypropylenes include polyoxyethylene polyoxypropylene glycol

(pluronic®) and polyoxyethylene polyoxypropylene cetyl ether.

【0047】

The suitable polyalcohols include glycerin (glycerol), propylene glycol, and monoglyceryl stearate; and the suitable anionic surfactants include alkyl ether sulfate such as sodium cetyl sulfate, sodium lauryl sulfate and sodium oleyl sulfate; alkyl sulfosuccinate such as sodium lauryl sulfosuccinate. The suitable synthetic or semi-synthetic polymers include polyvinyl alcohol, carboxyvinyl polymer, polyvinyl pyrrolidone and sodium polyacrylate.

【0048】

Examples of saccharides include monosaccharide such as mannitol, glucose, fructose, inositol, sorbitol, and xylitol; disaccharide such as lactose, sucrose, maltose, and trehalose; polysaccharide such as starch, dextran, pullulan, alginic acid, hyaluronic acid, pectinic acid, phytic acid, phytin, chitin, and chitosan. Examples of dextrin include α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, dextrin, hydroxypropyl starch, and hydroxyl starch. Examples of celluloses include methylcellulose, ethylcellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, and hydroxypropyl methylcellulose, sodium carboxymethyl cellulose.

【0049】

The suitable amino acids include glycine and taurine; and polyamino acid such as polyglutamic acid, polyaspartic acid, polyglycine and polyleucine. The Examples of proteins include albumin and gelatin.

【0050】

Non-human serum albumin can be used as anti-adsorbent for the pharmaceutical composition of the present invention when the composition is used as a reagent for examination or as veterinary medicines; however, it is preferable to use human serum albumin when the composition is used for a medicine for treating human being.

【0051】

These anti-adsorbents can be used in combination. The concentration

of the anti-adsorbent is in the range wherein the amount of the anti-adsorbent is pharmaceutically acceptable one and the adsorption of the ghrelins to the vessel is inhibited and the aggregation of the components does not occur during the manufacturing process or the long-term storage. For
5 example, the concentration of the anti-adsorbent is in the range of 0.001 to 5%, preferably from 0.01 to 1%.

【0052】

The pharmaceutical composition of the present invention can contain further additives for any purpose, and examples of the additives is selected
10 from the "Handbook of PHARMACEUTICAL EXCIPIENTS 2000" (Japan Pharmaceutical Excipients Council: Yakuji Nippoh Sha). These include isotonicizing agent such as sodium chloride and mannitol; antiseptic agent such as sodium benzoate; antioxidant such as sodium bisulfite, sodium pyrosulfite and ascorbic acid; soothing agent such as lidocaine hydrochloride and
15 mepivacaine hydrochloride.

【0053】

The manufacture of the pharmaceutical composition of the present invention is conducted by means of the common procedure applied in the pharmaceutical field. For example, first, freeze dried ghrelin is
20 dissolved in the purified water, and then, buffer agent, anti-adsorbent and other additives are also dissolved in another purified water. Then the resulting water solutions are combined and sterilized by filtration if necessary, and the obtained solution is filled in ampoules or vials to obtain the pharmaceutical composition containing the ghrelins of the
25 present invention.

【0054】

As the dosage form for the injectable preparation, there is in situ preparation for the pharmaceutical composition. This dosage form is suitable for the compound, which is unstable in the solution for long-term
30 storage. Therefore, the composition to prepare the solution containing the ghrelins in situ is one of the injectable preparations of the present invention. The composition to prepare the solution containing the ghrelins

in situ can contain raw material of the ghrelins for medicines and other additives with necessary amounts in a solid state. Further, the composition is obtained by drying the solution containing the ghrelins and other additives with necessary amounts. The dry technique of the solution can be the freeze-drying method or the spray drying method, and the freeze-drying method is preferred. These solid compositions can be used as the solution with water in situ.

【0055】

The pharmaceutical composition of the present invention can be administered to the mammal (human, monkey, dog, mouse and so on) as medicine.

The pharmaceutical composition of the present invention is administered by various kinds of administering route with an aqueous solution. For example, the pharmaceutical composition of the present invention is administered in the form of injectable solution such as intravenous injection, subcutaneous injection, intramuscular injection or intravenous drip. Further, the pharmaceutical composition of the present invention is administered by parenteral route such as nasal route, transpulmonary route, transdermic route or transmucosal route. Furthermore, the pharmaceutical composition of the present invention is parenterally administered in the form of ophthalmic solution or capsule filled with the solution.

【0056】

【Example】

The stability of the ghrelins of the present invention is illustrated in more detail by way of the following Tests and Examples, but it is to be noted that the present invention is not limited by those tests and examples in any way.

【0057】

In the following description, the following symbols are used to have the particular meanings and the following test methods and the instruments are used while it is not stated otherwise.

【0058】

[Symbols]

	Dha:	dehydroalanine
	TFA:	trifluoroacetic acid
5	HBTU:	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl- uronium hexafluorophosphate
	HOBt:	1-hydroxybenzotriazole
	TIPS:	tri-isopropylsilane
	DIPEA:	diisopropylethylamine
	Fmoc:	fluorenylmethoxycarbonyl
10	Boc:	t-butyloxycarbonyl
	tBu:	t-butyl
	Trt:	trityl
	DMAP:	4-dimethylaminopyridine
	EDC:	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
15	Pmc:	2,2,5,7,8-pentamethylchroman-6-sulfonyl

[0059]

[Instruments]

(A) Automatic peptide synthesizer

Applied Biosystems: Automatic 433A peptide synthesizer

20 (B) HPLC systems for analysis

Instrument: Shimadzu LC-10A system

Column: YMC-Pack PROTEIN-RP (4.6mmF x 150mm) or
YMC-Pack ODS-AM (4.6mmF x 250mm)

Column temperature: 40°C

25 Eluent: acetonitrile in 0.1% TFA, with linear gradient
of max 50% concentration.

Flow rate: 1mL/min

Detection: UV (210 nm)

Loaded volume: 10 to 500µL

30 [0060]

(C) HPLC systems for aliquot

Instrument: Waters 600 Multisolvant Delivery System

Column: YMC-Pack ODS-A (20mmF x 250mm) or

YMC-Pack PROTEIN-RP (20mmF x 250mm)

Eluent: acetonitrile in 0.1% TFA or 5% acetic acid, with linear gradient.

5 Flow rate: 10mL/min

Detection: UV (210 and 260nm)

Loaded volume: 1 to 2mL (more than 2mL, loaded by pump)

【0061】

(D) Storage chamber

10 Constant temperature and humidity chamber LH-30

(Nagano Kagaku) 5°C/40°C

Prefab type constant temperature and humidity chamber LH-20

(Nagano Kagaku) 25°C

【0062】

15 (E) Mass spectrum

Instrument: Finnigan MAT TSQ700

Ion source: ESI

Detective ion mode: positive

Spray voltage: 4.5kV

20 Capillary temperature: 250°C

Mobile phase: 0.2% acetic acid in H₂O/methanol (1/1)

Flow rate: 0.2mL/min

Scan area: m/z 300 to 1500

【0063】

25 (F) Analysis of amino acid sequence

Instrument: Applied Biosystem 477A Sequencer (Perkin-Elmyer)

(G) Analysis of amino acid composition

Instrument: Amino acid analyzer L-8500 (Hitachi)

Sample: Hydrolyzed with 6M-HCl containing 0.1% phenol

30 in sealed tube at 110°C for 24 hours.

【0064】

Reference Example: Synthesis of human ghrelin

Using the Automatic peptide synthesizer, Boc-Gly-Ser(tBu)-Ser(Trt)-Phe-Leu-Ser(tBu)-Pro-Glu(OtBu)-His(Boc)-Gln(Trt)-Arg(Pmc)-Val-Gln(Trt)-Gln(Trt)-Arg(Pmc)-Lys(Boc)-Glu(OtBu)-Ser(tBu)-Lys(Boc)-Lys(Boc)-Pro-Pro-Ala-Lys(Boc)-Leu-Gln(Trt)-Pro-Arg(Pmc)-HMP resin was synthesized from Fmoc-Arg(Pmc)-HMP-resin (Applied Biosystems Japan; 472 mg, 0.25 mmol) by repeated deletion of Fmoc and insertion of Fmoc-amino acid (provide that, Boc-glycine was used in the case of glycine N-terminal) by HBTU/HOBt. The obtained protected peptide-resin (1.7g) was treated with 1% TFA/5% TIPS/methylene chloride solution (15mL) for 30 minutes. The peptide-resin was collected and washed with methylene chloride (30mL) for several times, and with 1%DIPEA (30mL) then with methylene chloride (30mL). The obtained Trt deleted peptide-resin was swelled in N-methylpyrrolidone (10mL) and the mixture was reacted with octanoic acid (144.2mg, 1.0mmol) and EDC-HCl (192mg, 1.0mmol) in the presence of DNAP (31mg, 0.25mmol) for 16 hours. The resulting resin was collected by filtration and washed with N-methylpyrrolidone and methylene chloride respectively, and dried in vacuo to obtain protected peptide-resin wherein side chain of serine at 3-position was substituted by octanoyl group. Then, deprotection reagent consisting of 88% TFA/5% phenol/2% TIPS/5% H₂O (15mL) was added to the obtained resin and the mixture was stirred for 2 hours at room temperature. The resin was removed off by filtration and the filtrate was concentrated. The obtained residue was treated with ether to give the precipitate, and collected by filtration. The precipitate was dried to give 900mg of crude peptide. 200mg of the obtained crude peptide was dissolved in 10mL of water and the solution was added to YMC-Pack ODS-A column (20mmF x 250mm) and eluted by 5% TFA with linear gradient of 0 to 60% of acetonitrile for 60 minutes (flow rate: 10mL/min). The objective eluted parts were collected and lyophilized to give 60mg of target peptide (human ghrelin acetic acid salt: acetic acid content: 10.9%).

【0065】

ESI-MS: 3371 (calculated: 3370.9)

Leu standard amino acid composition: Ser; 3.43 (4), Glx; 5.93 (6), Gly;

Appln. Cert. Pat. 2003-3045624

1.01 (1), Ala; 1.00 (1), Val; 0.98 (1), Leu; 2 (2), Phe; 1.00 (1), Lys; 4.02 (4), His; 1.00 (1), Arg; 2.98 (3), Pro; 3.93 (4) (theoretical volume are in parentheses).

Analysis of amino acid sequence: The obtained peptide is identified with
5 human ghrelin (octanoyl-Ser at 3 position not detected).

【0066】

Rat ghrelin or other the ghrelins were obtained by using the same procedure mentioned above. In the following Example, the ghrelins obtained by the Reference Example was used.

10 【0067】

Example 1: Structural analysis of the degradation products of the ghrelins

It is necessary to know the degradation reaction of the ghrelins in the aqueous solution to secure the stability of ghrelin in the aqueous solution. Therefore, the degradation process of ghrelin was estimated
15 by the structural analysis of the degradation products of the ghrelins by using human ghrelin, which is one of the ghrelins.

【0068】

The aqueous solution containing about 0.15 $\mu\text{mol/mL}$ (0.5mg/mL) of human ghrelin was obtained by dissolving about 5.0 μmol (17mg) of human
20 ghrelin in Britton-Robinson buffer solution (pH 7.0: adjusted by 0.04M of phosphate/acetic acid/boric acid solution) and 0.2M sodium hydroxide aqueous solution. The obtained solution was filled in brownish glass ampoules and the ampoules were sealed with fire. The each ampoules were stored at $40 \pm 1^\circ\text{C}$ for 4 and 14 days. The degradation products in the aqueous
25 solutions after storage were detected by HPLC method and the results were shown in Figure 1 (a) and (b).

【0069】

As shown in Figure 1 (a), 2 major peaks (degradation product B and degradation product C) at 24 to 28 minutes were observed in the solutions
30 after stored at 40°C for 4 days. The two degradation product B and degradation product C were collected and the structural analysis of these degradation products from human ghrelin was conducted as follow.

【0070】

The degradation product B:

The mass of this product showed 3245 by ESI-MS analysis, and was identified with the mass of desacylated human ghrelin (hereinafter, referred to as "desacyl compound") obtained from the hydrolytic cleavage of octanoyl group of human ghrelin. Further, from the results of amino acid sequence and amino acid composition analysis of the degradation product B, the amino acid composition and the amino acid sequence were identified with the theoretical volume of those of desacyl compound. In conclusion, it was confirmed that the degradation product B was desacyl compound.

【0071】

The degradation product C:

The mass of this product showed 3227 by ESI-MS analysis, and was identified with the mass of [3-Dehydroalanine]human ghrelin (hereinafter, referred to as "Dha compound") obtained from the β -elimination of octanoyl group of human ghrelin. Further, from the results of ESI-MS, amino acid sequence and amino acid composition analysis of the product obtained by the degradation product C by reacting with excess ethanethiol in an aqueous solution neutralized with 0.05M sodium hydroxide aqueous solution, the product was identified with [3-Ethylcysteine]human ghrelin. That is, the mass of 3289 by ESI-MS was identified with the calculated addition value of Dha compound (3227) and ethanethiol (62), and ethylcysteine was detected from amino acid sequence and amino acid composition analysis. The product, [3-Ethylcysteine]human ghrelin, was obtained from Dha compound by nucleophilic reaction of ethanethiol. In conclusion, it was confirmed that the degradation product C was Dha compound.

【0072】

From the above-mentioned results, it was confirmed that the degradation products in the neutralized aqueous solution of human ghrelin, which is one of the ghrelins, were desacyl compound obtained from the hydrolytic cleavage of octanoyl group of human ghrelin and Dha compound obtained from the β -elimination of octanoyl group of human ghrelin.

【0073】

As shown in Figure 1 (b), the HPLC results of the solution stored for 14 days, several peaks (degradation product D, E and so on) were observed in addition to peaks of degradation product B and C. Then, the desacyl compound (degradation product B) and the Dha compound (degradation product C) were stored in aqueous solution to examine the mechanism of production of these degradation products.

【0074】

The desacyl compound or the Dha compound was dissolved in Britton-Robinson buffer solution (pH 7.0) to prepared an aqueous solution containing about 0.15 $\mu\text{mol/mL}$ (0.5mg/mL) of the desacyl compound or the Dha compound in same manner described above. The obtained solution was filled in brownish glass ampoules and the ampoules were sealed with fire. The ampoules filled with desacyl compound were stored at $40\pm 1^\circ\text{C}$ for 14 days, and the ampoules filled with Dha compound were storage at $40\pm 1^\circ\text{C}$ for 3 days. The degradation products in the aqueous solutions after stored were detected by HPLC method and the results were shown in Figure 1 (c) and (d).

【0075】

As shown in Figure 1 (c), one major peak at the same retention time (26 minutes) as that of degradation product D in Figure 1 (b) was observed in the solutions containing desacyl compound, after stored at 40°C for 14 days. The degradation product at this peak was collected and ESI-MS, amino acid sequence and amino acid composition analysis were conducted. From the results of these analyses, this product was identified with desacyl human ghrelin (3-28). ESI-MS: 3101 (calculated value: 3100.5).

【0076】

Further, as shown in Figure 1 (d), one broad peak at the same retention time (36 minutes) as that of degradation product E in Figure 1 (b) was observed in the solutions containing Dha compound, after stored at 40°C for 3 days. The degradation product at this peak was collected and ESI-MS, amino acid sequence and amino acid composition analysis were conducted.

From the results of these analyses, this product was estimated to be [N^a-CO-C(=CH₂)-OH]human ghrelin(4-28) (hereinafter, referred to as "2nd degradation product of Dha compound"). ESI-MS: 3083 (calculated value: 3083.5), Amino acid composition: Identified with the estimated amino acid composition. Amino acid sequence: No reaction from first amino acid residue. Furthermore, in Figure 1 (d), several small peaks were observed at about 30 to 35 minutes. These peaks were also observed in Figure 1 (b), and it was estimated that these degradation products were produced from human ghrelin via Dha compound.

10 【0077】

From the above-mentioned results, the degradation process of the aqueous solution containing human ghrelin was confirmed that the desacyl compound or the Dha compound was produced from human ghrelin at the beginning, and then, the desacyl human ghrelin (3-28) was produced by fragmentation of the desacyl compound and 2nd degradation product of Dha compound and further products were produced by fragmentation of the desacyl compound, respectively.

15 【0078】

Therefore, it was confirmed that to obtain the stability of the ghrelins in an aqueous solution, it was necessary to prevent the various type of cleavage reactions at the hydrophobic group, which was the characteristic structure of the ghrelins.

20 【0079】

Example 2: Stability of the ghrelins in buffer solutions having various kinds of pH value (Stability Test 1)

25 The influence of pH value of the solution containing the ghrelins was conducted using human ghrelin, which is one of the ghrelins.

Human ghrelin was dissolved in the following aqueous solutions in the concentration of about 0.15 μ mol/mL (0.5mg/mL).

30 0.1M HCl aqueous solution (pH: 1.1)

McIlvain buffer solutions (pH: 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0)

The pH was adjusted with 0.1M citric aqueous acid and 0.2M dibasic

sodium phosphate aqueous solution.

Each solution were stored at $25 \pm 2^\circ \text{C}$ for 8, 24, 48 and 72 hours respectively, and the obtained solutions were detected by HPLC analysis in comparison to the solutions before storage. The peak area ratio of
5 human ghrelin, desacyl compound and Dha compound to the total area were calculated. No significant changes of pH in solution before storage and after storage were observed. The results were shown in Table 2.

【0080】

【Table 2】

10 Table2

		pH observed	Peak area ratio to total peak area (%)		
			Human ghrelin ^{a)}	Desacyl compound ^{b)}	Dha compound ^{b)}
pH 1	Before	1.1	98.89	0.27	0.21
	8 hr. after	1.2	<u>94.40</u>	<u>4.88</u>	0.15
	24 hr. after	1.2	<u>86.00</u>	<u>13.31</u>	0.09
	48 hr. after	1.2	<u>74.62</u>	<u>24.63</u>	0.04
	72 hr. after	1.2	65.04	34.17	0.00
pH 2	Before	2.0	99.07	0.10	0.20
	8 hr. after	2.0	98.82	0.34	0.20
	24 hr. after	2.0	98.24	0.89	0.23
	48 hr. after	2.0	<u>97.35</u>	<u>1.76</u>	0.24
	72 hr. after	2.0	96.46	2.59	0.25
pH 3	Before	3.0	99.07	0.10	0.20
	8 hr. after	3.1	98.98	0.20	0.20
	24 hr. after	3.1	98.77	0.38	0.23
	48 hr. after	3.1	98.40	0.67	0.24
	72 hr. after	3.1	98.03	0.98	0.25
pH 4	Before	4.0	99.07	0.09	0.20
	8 hr. after	4.0	99.07	0.12	0.20
	24 hr. after	4.0	98.92	0.21	0.21
	48 hr. after	4.0	98.69	0.37	0.22
	72 hr. after	4.0	98.44	0.51	0.23
pH 5	Before	5.0	99.06	0.10	0.21
	8 hr. after	5.0	99.04	0.14	0.21
	24 hr. after	5.0	98.91	0.24	0.21
	48 hr. after	5.0	98.59	0.45	0.23
	72 hr. after	5.0	98.37	0.61	0.24
pH 6	Before	6.0	99.07	0.10	0.20
	8 hr. after	6.0	98.87	0.28	0.21
	24 hr. after	6.0	98.28	0.70	0.26
	48 hr. after	6.0	<u>97.64</u>	<u>1.33</u>	0.31
	72 hr. after	6.0	96.92	1.97	0.35
pH 7	Before	7.0	98.97	0.16	0.22
	8 hr. after	7.0	98.15	0.92	0.25
	24 hr. after	7.0	<u>96.18</u>	<u>2.70</u>	0.43
	48 hr. after	7.0	<u>93.44</u>	<u>5.22</u>	0.61
	72 hr. after	7.0	90.75	7.67	0.79
pH 8	Before	8.0	98.61	0.47	0.24
	8 hr. after	7.9	<u>94.73</u>	<u>3.92</u>	0.68
	24 hr. after	7.9	<u>87.32</u>	<u>10.57</u>	<u>1.39</u>
	48 hr. after	7.9	<u>76.95</u>	<u>19.54</u>	<u>2.39</u>
	72 hr. after	7.9	68.30	27.26	3.12

【0081】

a) The place where ratio of peak area of human ghrelin is below 98% is under lined.

b) The place where ratio of peak area of desacyl compound or Dha compound
5 below 1% is under lined.

【0082】

As shown in Table 2 above, more than 3% of the desacyl compound was produced in the aqueous solution of human ghrelin at pH 1.0 and 8.0 in the shortest time, such as storage for 8 hours. Further, more than
10 1% of the desacyl compound was produced in the aqueous solution of human ghrelin at pH 7.0 stored for 24 hours and at pH 2.0 and 6.0 stored for 48 hours, and ratio of human ghrelin was less than 98%. On the contrary, the production of the desacyl compound and the Dha compound was inhibited in the solution having the pH range of 3.0 to 5.0.

15 【0083】

It was understood that the aqueous solution having the pH range of 2 to 7, preferably 3 to 6 was suitable for inhibiting the production of the desacyl compound and the Dha compound. Therefore, it was confirmed that to obtain the stability of the ghrelins in an aqueous solution, it
20 was necessary to adjust the pH of the solution to the range of 2 to 7, preferably 3 to 6 to prevent the various type of cleavage reactions at the hydrophobic group, which is the characteristic structure of the ghrelins.

【0084】

25 Example 3: Stability of the ghrelins in buffer solutions having various kinds of pH value (Stability Test 2)

Using different buffer solution from the buffer solution of the Example 2, the stability of the ghrelins was conducted in the buffer solution having various kinds of pH value.

30 Human ghrelin, which is one of the ghrelins, was dissolved in the Britton-Robinson buffer solutions which is adjusted by combining 0.04M phosphoric acid-acetic acid-boric acid aqueous solution and 0.2M sodium

hydrate aqueous solution in appropriate ratio, having pH of 2.1, 3.1, 4.0, 5.0, 6.0, 7.0 and 7.9 to obtain an aqueous solution containing human ghrelin in the concentration of about 0.15 $\mu\text{mol/mL}$ (0.5mg/mL). The obtained solution was filled in brownish glass ampoules and the ampoules were sealed
5 with fire. For the calculation of kinetic constant, the certain degrees of degradation products have to be occurred in the aqueous solution of the ghrelins, and therefore, the ampoules were stored at $40 \pm 1^\circ\text{C}$, which was severe conditions for storage. The concentration (residual ratio) of human ghrelin was conducted by HPLC with time. At the same time, the
10 pH of the solution was measured and it was confirmed that there was no significant changes of pH in solution.

The kinetic constants in each pH solution were calculated from the sequential change of the residual ratio, and the results were shown in Figure 2.

15 Further, the peak area ratios of desacyl compound and Dha compound to the total area in each solution having various kinds of pH, after stored at 40°C for 1 day, were shown in Figure 3.

[0085]

As shown in Figure 2, it was confirmed that the pH range of the
20 solution stably containing human ghrelin was from 3 to 6. The residual ratios of ghrelin in the solutions having the pH of 3.1, 4.0, 5.0 and 6.0 were more than 87%, and these value were exceeded the target value (85%).

Further, as shown in Figure 3, the production of desacyl compound
25 was inhibited in the solution having the pH range of 3 to 6, and the production of Dha compound was inhibited in the solution having the pH range of 2 to 7.

[0086]

From the results mentioned above, it was understood that in the
30 case of using Britton-Robinson buffer solution, the aqueous solution having the pH range of 2 to 7, preferably 3 to 6 was suitable for inhibiting the production of the desacyl compound and the Dha compound.

【0087】

Therefore, it was confirmed that to obtain the stability of the ghrelins in an aqueous solution, it was necessary to adjust the pH of the solution to 2 to 7, preferably 3 to 6 to prevent the various type of cleavage reactions at the hydrophobic group, which was the characteristic structure of the ghrelins.

【0088】

Example 4: The influence of the varieties of buffer solution for stability of the ghrelins (Test 1)

The stability of the ghrelins by using citrate buffer solution was examined.

Human ghrelin, which is one of the ghrelins, was dissolved in the citrate buffer solutions having pH of 3.6, 4.0, 4.5 and 5.0 to obtain an aqueous solution containing human ghrelin in the concentration of about 0.15 μ mol/mL (0.5mg/mL). The obtained solutions were filled in brownish glass ampoules and the ampoules were sealed with fire, then, each ampoules were stored at 40 \pm 1°C for 2 weeks. After storage for 2 weeks, the HPLC analysis of the solutions was conducted to calculate the residual ratio from the concentration of human ghrelin. The change of pH value was also measured.

These results were summarized in the following Table 3.

【0089】

【Table 3】

Table 3:

pH of buffer solution		Just after preparation	After storage for 40° C/2 weeks
3.5	Residual ratio (%)	100	89
	pH (observed)	3.6	3.5
4.0	Residual ratio (%)	100	88
	pH (observed)	4.0	4.0
4.5	Residual ratio (%)	100	86
	pH (observed)	4.5	4.4
5.0	Residual ratio (%)	100	85
	pH (observed)	5.0	4.9

【0090】

As shown in Table 3, the residual ratios of human ghrelin were from 85 to 89% when human ghrelin was dissolved in citrate buffer solutions having the pH range of 3.5 to 5.0, and these values exceeded the target value (85%). Further, no significant changes of pH in solution before storage and after storage were observed. Therefore, it was confirmed that the stability of the ghrelins in citrate buffer solution was obtained in the pH range of 3.5 to 5.0.

【0091】

10 Example 5: The influence of the varieties of buffer solution for stability of the ghrelins (Test 2)

The stability of the ghrelins was examined by using glycine hydrochloride buffer solution or acetate buffer solution.

Human ghrelin, which is one of the ghrelins, was dissolved in the 0.05M glycine hydrochloride buffer solutions having pH of 2.5, 3.1, 3.6, 4.2, 4.6 and 4.8, or in the 0.05M acetate buffer solution having pH of 3.1, 3.5, 4.0, 4.5 and 5.0, to obtain an aqueous solution containing human ghrelin in the concentration of about 0.15 μ mol/mL (0.5mg/mL). The obtained solutions were filled in brownish glass ampoules and the ampoules were sealed with fire, then, each ampoules were stored at 40 \pm 1°C for 2 weeks. After storage for 2 weeks, the HPLC analysis of the solutions was conducted to calculate the purity. The change of pH value was also examined to confirm no significant changes of pH of solution occurred.

25 The residual ratios of human ghrelin in the each buffer solution of various kinds of pH after storage for 2 weeks were summarized in Figure 4.

As shown in Figure 4, the residual ratios of human ghrelin were from 87 to 97% when human ghrelin was dissolved in glycine hydrochloride buffer solution or acetate buffer solution having the pH range of 2.5 to 5.0, and these values exceeded the target value (85%).

【0092】

From the results of the Example 2 (stability in McIlvaine buffer

solution), the Example 3 (stability in Britton-Robinson buffer solution), the Example 4 (stability in citrate buffer solution), and the Example 5 (stability in glycine hydrochloride or acetate buffer solution), it was confirmed that the pH adjustment of the solution was important to obtain the stability of the ghrelins in aqueous solution, and the variety of buffer solution had no effect on the stability of the ghrelins in aqueous solution.

【0093】

Example 6: Stability of the ghrelins in aqueous solution with various kinds of concentrations

The stability of the ghrelins in aqueous solution with various kinds of concentration was examined.

Human ghrelin, which is one of the ghrelins, was dissolved in the 0.05M glycine hydrochloride buffer solutions (pH 3.5) to obtain an aqueous solution containing human ghrelin with following five concentrations, which are applicable for medical usage.

0.03nmol/mL (0.1 μ g/mL); 0.3nmol/mL (1.0 μ g/mL);

3.0nmol/mL (10.0 μ g/mL); 0.3 μ mol/mL (1.0mg/mL);

3 μ mol/mL (10mg/mL).

Each solutions were stored at $25 \pm 2^{\circ}$ C for 24 hours, and after storage, the HPLC analyses of each solutions were conducted to calculate the residual ratios from the concentration of human ghrelin. The changes of pH value were also examined to confirm no significant changes of pH in solutions occurred.

The residual ratio of the solution before storage was referred to as 100%, and the residual ratio of each solution after storage and the changes of pH value of each solution were summarized in the following Table 4.

【0094】

【Table 4】

Table 4:

Concentration of h.ghrelin		Just after preparation	After storage for 25° C/24 hours
0.03nmol/mL	Residual ratio (%)	100	62
	pH	3.4	3.5
0.3nmol/mL	Residual ratio (%)	100	98
	pH	3.5	3.5
3.0nmol/mL	Residual ratio (%)	100	98
	pH	3.5	3.6
0.3µmol/mL	Residual ratio (%)	100	101
	pH	3.4	3.3
3µmol/mL	Residual ratio (%)	100	101
	pH	3.4	3.3

【0095】

As shown in Table 4, the solutions with high concentration of human ghrelin (0.3nmol/mL, 3.0nmol/mL, 0.3µmol/mL and 3µmol/mL) kept the stability of human ghrelin in the aqueous solutions during the storage at 25° C for 24 hours, and no decrease of the residual ratios and the changes of pH value were observed.

On the contrary, in the case of the solution with low concentration of human ghrelin (0.03nmol/mL), the residual ratio of the solution after storage was 62%; however, no significant change of pH value was observed and there was no degradation product such as desacyl compound or Dha on the HPLC chart analysis. Therefore, it was confirmed that the decrease of the residual ratio of human ghrelin in the solution might be the decrease of the content of human ghrelin due to its adsorption to the wall of the vessel. Accordingly, it was decided that the solutions with low concentration of human ghrelin (0.03nmol/mL) also kept the stability of human ghrelin in the aqueous solution during the storage at 25° C for 24 hours.

In conclusion, it was confirmed that the ghrelins were stably contained in the pH adjusted buffer solution in the concentration range of about 0.03nmol/mL to about 3µmol/mL.

【0096】

Example 7: Adsorption inhibiting effect of anti-adsorbents in aqueous

solution containing the ghrelins with low concentration

In the Example 6, it was shown that the ghrelins adhere to the wall of the vessel in the solution having medical applicable concentration of the ghrelins. Accordingly, the adsorption inhibiting effect of anti-adsorbents in aqueous solution containing the ghrelins was examined.

As the anti-adsorbent, the surfactant, that is, polyoxyethylene sorbitan monooleate (hereinafter, referred to as Tween® 80), and benzalkonium chloride were selected.

Human ghrelin was dissolved in 5% mannitol-0.05M glycine hydrochloride buffer solution (pH 3.5) with about 0.3nmol/mL (1.0µg/mL) concentration, which was the medical applicable concentration of the ghrelins. The anti-adsorbent was added to this solution with 0.01% or 0.1% concentration.

【0097】

The human ghrelin concentrations of each solution were measured by HPLC method right after preparation, and these solutions were replaced in glass test tubes. After that, each solution were further replaced in new glass test tubes, and same operation was repeated for 5 and 10 times, then, the human ghrelin concentrations of each treated solution were measured by HPLC method. As a control, the solution not containing anti-adsorbent was examined.

Same procedure was repeated by using test tube made by polypropylene instead of glass test tube.

The results were summarizes in Table 5.

【0098】

【Table 5】

Table 5:

Anti-adsorbent		Initial Conc.	Glass tube ^{a)}		Polypropylene tube ^{b)}	
Species	Conc.		5 times	10 times	5 times	10 times
None	-	100	0	0	51	19
Tween 80	0.01%	100	17	0	90	90
	0.1%	100	20	5	93	91
benzalkonium chloride	0.01%	100	81	64	97	96
	0.1%	100	99	99	99	101

【0099】

a) Asahi Techno-glass Co., Ltd.: 10mL

b) CORNING Co., Ltd.: 15mL

5 【0100】

As clearly shown in Table 5, the human ghrelin concentrations of the solutions were greatly reduced after the replacements of the solution in both cases using glass tubes and polypropylene tubes. Particularly, in the case of using glass tubes, the human ghrelin concentration of the solution was reduced to the level not detected by the HPLC analysis.

On the contrary, high adsorption inhibiting effect against polypropylene tube was observed when Tween® 80 was added to the solution with 0.01% or 0.1% concentration, as anti-adsorbent, and also high adsorption inhibiting effect against both glass tube and polypropylene tube was observed when benzalkonium chloride was added to the solution as anti-adsorbent.

【0101】

Accordingly, it was well understood that Tween® 80 and benzalkonium chloride have a beneficial effect on inhibiting the ghrelins adsorption to the wall of the vessel, of the solution containing the ghrelins with the medical applicable concentration. Therefore, it was confirmed that the anti-adsorbent was effective to prevent the adsorption of the ghrelins during the manufacturing process, long-term storage and administering process.

25 【0102】

Example 8: Manufacture of the in situ preparation for solution and its

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stability

As the in situ preparation for the pharmaceutical composition of the present invention, lyophilized powder was prepared and the solubility of the powder was estimated.

5 Human ghrelin was dissolved in 5% mannitol-0.05M glycine hydrochloride buffer solution (pH 3.5) with about 0.3nmol/mL (1.0 μ g/mL) concentration, and the human ghrelin concentration of this solution just after preparation was measured by HPLC method, and pH value of this solution was also measured. As the results, the human ghrelin concentration was 10 1.0 μ g/mL, and pH was 3.5. Then, this solution was lyophilized at -25°C for 24 hours in vacuo, and the obtained powder was further dried at 20°C for 24 hours in vacuo. The obtained lyophilized powder was white solid having good figure.

【0103】

15 Then, this lyophilized powder was dissolved in the purified water, which amount was same as the decreased amount from the beginning of the lyophilization, and the solubility of the lyophilized powder and the pH of the obtained solution were examined. As the result, the solubility of the lyophilized powder was excellent and no insoluble matter appeared 20 in the solution, and the solubility of the powder was suitable for the in situ preparation for solution. The human ghrelin concentration was 1.0 μ g/mL, which is same human ghrelin concentration of the solution just after the preparation or before the lyophilization, therefore, no decrease of content of the preparation by lyophilization was observed. Further, 25 the pH value of the solution was 3.5, which was same pH value of the solution just after the preparation or before the lyophilization. Therefore, the aqueous solution stably containing human ghrelin can be obtained from the lyophilized powder of the present invention.

【0104】

30 Accordingly, the lyophilized powder containing the ghrelins can be obtained by using the solution containing the ghrelins with adjustment of pH of the solution by lyophilization, and obtained lyophilized powder

preparation is useful for the in situ preparation for the pharmaceutical composition of the present invention.

【0105】

Manufacturing Example 1: Preparation of pharmaceutical composition
5 containing human ghrelin with pH adjustment

Human ghrelin, which is one of the ghrelins, was dissolved in purified water to obtain an aqueous solution containing human ghrelin with about 0.15 μ mol/mL (0.5mg/mL). The pH of this solution was adjusted to 4.0 by adding 0.1M hydrochloride acid solution, to obtain the pharmaceutical
10 composition containing human ghrelin as the solution preparation.

【0106】

Manufacturing Example 2: Preparation of pharmaceutical composition
containing rat ghrelin in glycine hydrochloride buffer solution

Rat ghrelin, which is one of the ghrelins, was dissolved in 0.05M
15 glycine hydrochloride buffer solution (pH 3.5) with about 0.15 μ mol/mL (0.5mg/mL) to obtain the pharmaceutical composition containing rat ghrelin as the solution preparation. The pH of this solution was 3.5.

【0107】

Manufacturing Example 3: Preparation of pharmaceutical composition
20 containing [3-Serine(acetyl)]human ghrelin in glycine hydrochloride
buffer solution

[3-Serine(acetyl)]human ghrelin, which is one of the ghrelins, was dissolved in 0.05M glycine hydrochloride buffer solution (pH 3.5) with about 0.15 μ mol/mL (0.1mg/mL) to obtain the pharmaceutical composition
25 containing [3-Serine(acetyl)]human ghrelin as the solution preparation. The pH of this solution was 3.5.

【0108】

Manufacturing Example 4: Preparation of pharmaceutical composition
containing [3-Serine(phenylpropionyl)]human ghrelin in glycine
30 hydrochloride buffer solution

[3-Serine(phenylpropionyl)]human ghrelin, which is one of ghrelins, was dissolved in 0.05M glycine hydrochloride buffer solution (pH 3.5)

with about 0.15 μ mol/mL (0.5mg/mL) to obtain the pharmaceutical composition containing [3-Serine(phenylpropionyl)]human ghrelin as the solution preparation. The pH of this solution was 3.5.

【0109】

5 Manufacturing Example 5: Preparation of pharmaceutical composition containing human ghrelin(1-5)amide in glycine hydrochloride buffer solution

Human ghrelin(1-5)amide, which is one of ghrelins, was dissolved in 0.05M glycine hydrochloride buffer solution (pH 3.5) with about 10 0.15 μ mol/mL (0.5mg/mL) to obtain the pharmaceutical composition containing human ghrelin(1-5)amide as the solution preparation. The pH of this solution was 3.5.

【0110】

【Industrial Applicability】

15 As described above, the present invention provides the pharmaceutical composition stably containing the ghrelins, and the preparation of the present invention prevents the adsorption of ghrelins to the wall of the vessel. Therefore, the present invention provides the pharmaceutical composition without decrease of the ghrelins, during the 20 manufacture, long-term storage or administration.

【0111】

【SEQUENCE LISTING】

SEQUENCE LISTING

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<120> Pharmaceutical Composition Containing Ghrelin
<130> SN318
<160> 26
<170> PatentIn version 3.1
30 <210> 1
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<213> Homo sapiens

<223> Amino acid sequence for human endogenous peptides of growth hormone secretagogue

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1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20 25

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<211> 27

<212> PRT

<213> Homo sapiens

<223> Amino acid sequence for human endogenous peptides (27 amino acids)

15 of growth hormone secretagogue

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1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20 20 25

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25 <213> Rattus norvegicus

<223> Amino acid sequence for rat endogenous peptides of growth hormone secretagogue

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30 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20 25

<210> 4

<211> 27

<212> PRT

5 <213> Rattus norvegicus

<223> Amino acid sequence for rat endogenous peptides of growth hormone secretagogue

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10 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20 25

<210> 5

15 <211> 28

<212> PRT

<213> Mus musculus

<223> Amino acid sequence for mouse endogenous peptides of growth hormone secretagogue

20 <400> 5

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys

1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20 25

25

<210> 6

<211> 28

<212> PRT

<213> Sus scrofa

30 <223> Amino acid sequence for porcine endogenous peptides of growth hormone secretagogue

<400> 6

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Val Gln Gln Arg Lys

1 5 10 15

Glu Ser Lys Lys Pro Ala Ala Lys Leu Lys Pro Arg

5 20 25

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10 <213> Bos taurus

<223> Amino acid sequence for bovine endogenous peptides (27 amino acids)
of growth hormone secretagogue

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Ala Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg

20 25

<210> 8

20 <211> 27

<212> PRT

<213> Ovis aries

<223> Amino acid sequence for ovine endogenous peptides (27 amino acids)
of growth hormone secretagogue

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1 5 10 15

Pro Lys Lys Pro Ser Gly Arg Leu Lys Pro Arg

20 25

30

<210> 9

<211> 28

<212> PRT

<213> *Canis familiaris*

<223> Amino acid sequence for dog endogenous peptides of growth hormone secretagogue

5 <400> 9

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln Arg Lys

1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

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<210> 10

<211> 21

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<213> *Anguilla japonica*

15 <220>

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1 5 10 15

Lys Pro Pro Arg Val

20

25

<210> 11

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<212> PRT

<213> *Oncorhynchus mykiss*

30 <220>

<221> AMIDATION

<222> 23

<223> Amino acid sequence for rainbow trout endogenous peptides (23 amino acids) of growth hormone secretagogue.

<400> 11

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5 1 5 10 15

Lys Gly Lys Pro Pro Arg Val

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10 <211> 20

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<213> Oncorhynchus mykiss

<220>

<221> AMIDATION

15 <222> 20

<223> Amino acid sequence for rainbow trout endogenous peptides (20 amino acids) of growth hormone secretagogue.

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20 1 5 10 15

Pro Pro Arg Val

20

<210> 13

25 <211> 24

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<213> Gallus domesticus

<223> Amino acid sequence for chicken endogenous peptides of growth hormone secretagogue

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1 5 10 15

Gly Thr Arg Lys Pro Thr Ala Arg

20

<210> 14

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<213> Gallus domesticus

<223> Amino acid sequence for chicken endogenous peptides of growth hormone secretagogue

10 <400> 14

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1

5

10

15

Asp Thr Arg Lys Pro Thr Ala Arg

20

15

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<213> Gallus domesticus

20 <223> Amino acid sequence for chicken endogenous peptides of growth hormone secretagogue

<400> 15

Gly Ser Ser Phe Leu Ser Pro Thr Tyr Lys Asn Ile Gln Gln Gln Lys

1

5

10

15

25 Asp Thr Arg Lys Pro Thr Ala Arg Leu His

20

25

<210> 16

<211> 27

30 <212> PRT

<213> Rana catesbeiana

<223> Amino acid sequence for frog endogenous peptides of growth hormone

secretagogue

<400> 16

Gly Leu Thr Phe Leu Ser Pro Ala Asp Met Gln Lys Ile Ala Glu Arg

1 5 10 15

5 Gln Ser Gln Asn Lys Leu Arg His Gly Asn Met

20 25

<210> 17

<211> 28

10 <212> PRT

<213> Rana catesbeiana

<223> Amino acid sequence for frog endogenous peptides of growth hormone secretagogue

<400> 17

15 Gly Leu Thr Phe Leu Ser Pro Ala Asp Met Gln Lys Ile Ala Glu Arg

1 5 10 15

Gln Ser Gln Asn Lys Leu Arg His Gly Asn Met Asn

20 25

20 <210> 18

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<213> Tilapia nilotica

<221> AMIDATION

25 <223> Amino acid sequence for tilapia endogenous peptides of growth hormone secretagogue.

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1 5 10 15

30 Ser Ser Arg Ile

20

<210> 19

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<212> PRT

<213> Silurus asotus

5 <221> AMIDATION

<223> Amino acid sequence for catfish endogenous peptides of growth hormone secretagogue. This peptide is amidated at C-terminus.

<400> 19

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10 1 5 10 15

Arg Lys Pro Pro Arg Val

20

<210> 20

15 <211> 23

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<213> Silurus asotus

<223> Amino acid sequence for catfish endogenous peptides of growth hormone secretagogue

20 <400> 20

Gly Ser Ser Phe Leu Ser Pro Thr Gln Lys Pro Gln Asn Arg Gly Asp

1 5 10 15

Arg Lys Pro Pro Arg Val Gly

20

25

<210> 21

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<212> PRT

<213> Equus caballus

30 <223> Amino acid sequence for equine endogenous peptides of growth hormone secretagogue

<400> 21

1

10

15

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25

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【Brief Description of Drawings】

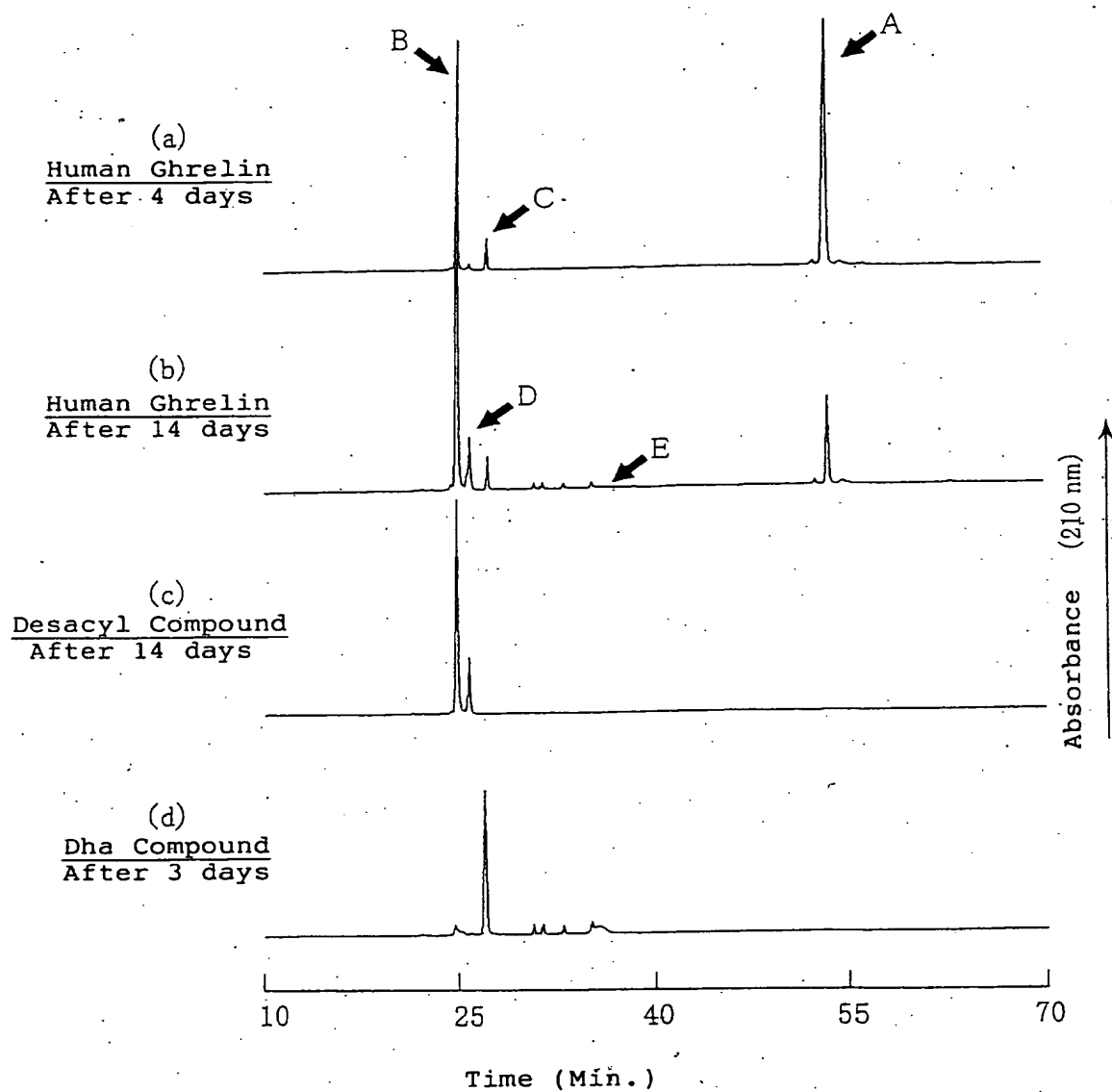
【Figure 1】 Figure 1 shows the HPLC chart regarding the degradation patterns of desacyl compound and Dha compound in an aqueous solution of the ghrelin in Example 1.

10 **[Figure 2]** Figure 2 is the graphic chart showing the profile of
pH-degradation kinetic constant of the qhrelins.

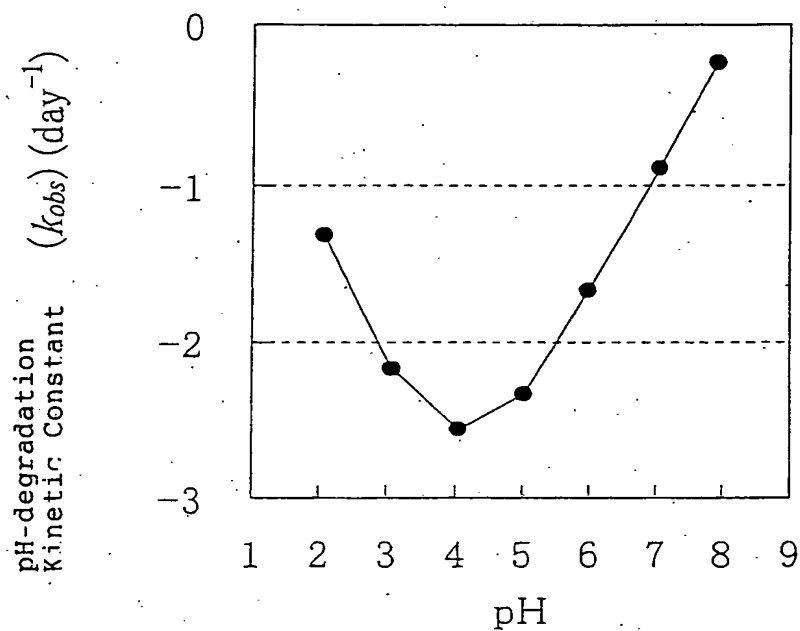
[Figure 3] Figure 3 is the graphic chart showing the production amount of the degradation product of the ghrelin in aqueous solution having various kinds of pH.

15 **[Figure 4]** Figure 4 is the graphic chart showing pH stability of the ghrelin
in various kinds of buffer solution.

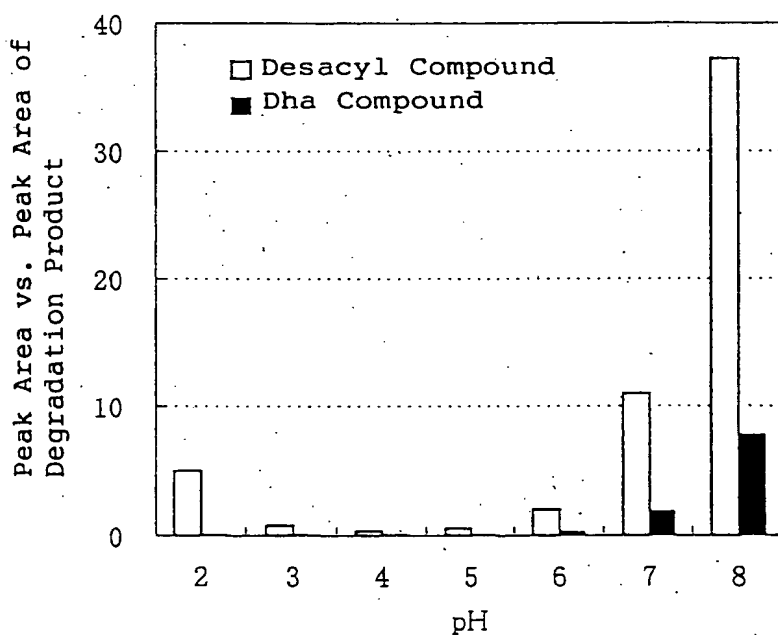
【Name of the Document】 Figure
【Fig.1】



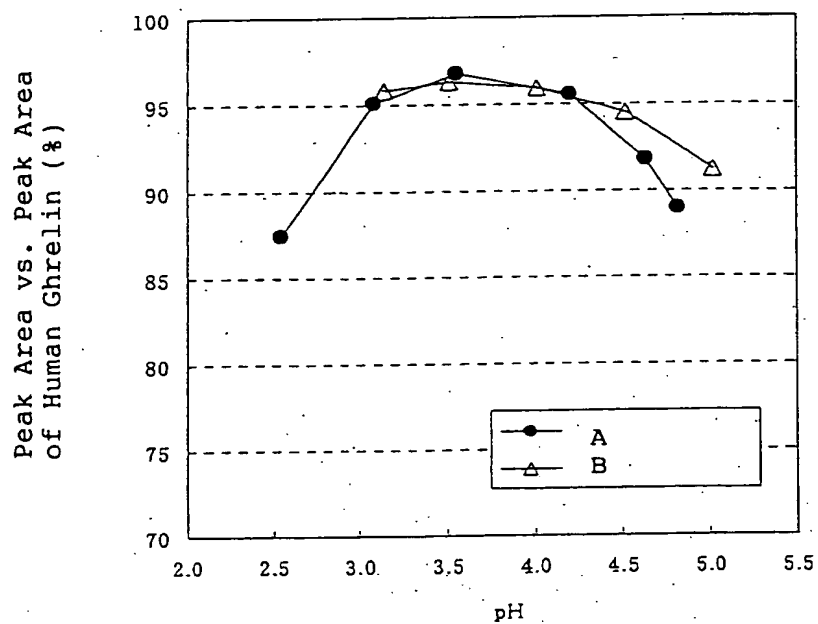
【Fig.2】



【Fig.3】



【Fig.4】



A: Glycine Hydrochloride Buffer
B: Acetate Buffer

【Name of the Document】 ABSTRACT

【Abstract】

5 【Purpose】 It is provided a pharmaceutical composition stably containing ghrelin or its derivative, which is an endogenous growth hormone secretagogue (GHS) to a growth hormone secretagogue-receptor (GHS-R).

10 【Means to solve the problem】 It is provided a pharmaceutical composition stably containing ghrelin or its derivative comprising a aqueous solution containing the ghrelins having pH range of 2 to 7, wherein the aqueous solution having pH range of 2 to 7 is a buffer solution, especially, glycine hydrochloride buffer, acetate buffer, citrate buffer, lactate buffer, phosphate buffer, citric acid-phosphate buffer, phosphate-acetate-borate buffer or phthalate buffer, and the concentration of the ghrelins in the solution is from 0.03nmol/mL to 6μmol/mL.

15 【Selected Figure】 None

JP2002-146155

Approval and Addition Information

Patent Application # Patent Application # 2002-146155

Reference Number 50200727021

5 Name of Document Application for Patent

Responsible OfficerNo.4 Upper Class 0093

Date Heisei 14 May 22

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10 [Date of Submission] Heisei 14 May 21

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[Name of Document] Change of the Applicant (General Transfer)

[Reference No.] SN318

[Addresses] To Commissioner of the Patent Office

[Identification of the Case]

5 [Application No.] JP2002-146155

[Person Transferred]

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[List of the Documents]

15 [Item] Corporation Demerger Liner Certificate 1

[claim] The certificate attached to Request for Transfer of Patent
Right of Patent No. 1770323 submitted on March 17, 2003 is claimed.

[Item] Company Register 1

[claim] The certificate attached to Request for Transfer of Patent
20 Right of Patent No. 1770323 submitted on March 17, 2003 is claimed.

[Item] Power of Attorney 1

[claim] The certificate attached to Request for Transfer of Patent
Right of Patent No. 1770323 submitted on March 17, 2003 is claimed.

[Proof] Requested

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Reference Number 50300428074
5 Name of Document Change of the Applicant (General Transfer)
Responsible Officer AMAMIYA, Masaaki 7743
Date Heisei 15 April 24

<Approval Information and Addition Information >

10 [Date of Submission] Heisei 15 March 17

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Appln. Cert. Pat. 2003-3045624

JP2002-146155

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JP2002-146155

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